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Immunoproteasomes Shape the Transcriptome and Regulate the Function of Dendritic Cells

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By regulating protein degradation, constitutive proteasomes (CPs) control practically all cellular functions. In addition to CPs, vertebrates express immunoproteasomes (IPs). The major nonredundant role ascribed to IPs is their enhanced ability to generate antigenic peptides. We report that CPs and IPs differentially regulate the expression of >8000 transcripts in maturing mouse dendritic cells (DCs) via regulation of signaling pathways such as IFN regulatory factors, STATs, and NF-kB. IPs regulate the transcription of many mRNAs and maturation of a few of them. Moreover, even when engineered to present optimal amounts of antigenic peptide, IP-deficient DCs are inefficient for in vivo T cell priming. Our study shows that the role of IPs in DCs is not limited to Ag processing and reveals a major nonredundant role for IPs in transcription regulation. The dramatic effect of IPs on the transcriptional landscape could explain the various immune and nonimmune phenotypes observed in vertebrates with IP deficiency or mutations. The Journal of Immunology, 2014, 193: 1121–1132.

In all eukaryotes, proteolysis of a variety of cellular proteins by constitutive proteasomes (CPs) plays an important role in many basic cellular processes including regulation of cell cycle and division, differentiation and development, morphogenesis, and response to stress (1). Vertebrates also express immunoproteasomes (IPs), in which the catalytic θ-subunits are replaced by IFN-γ-inducible homologs: LMP2 (aka β1i, Psmb9) for β1 (Psmb6), MECL1 (aka β2i, Psmb10) for β2 (Psmb7), and LMP7 (aka β5i, Psmb8) for β5 (Psmb5). The best-described nonredundant role ascribed to IPs is their enhanced ability to generate MHC class I–associated peptides (MIPs) (2–5).

As regulators of protein degradation, proteasomes regulate practically all cellular functions (1, 6, 7). It is therefore logical to assume that replacement of CPs by IPs could have pleiotropic and far reaching effects on cell function (8, 9). In line with this, deletion or inhibition of IP subunits can affect several immune cell functions independently of MIP processing: T cell proliferation, B cell survival, and cytokine production (10, 11). Moreover, the expression of IPs in numerous nonimmune cells from noninflamed tissues suggests that the roles of IPs are not limited to the immune system. Thus, IPs have been implicated in adipocyte differentiation (12), maintenance of pluripotency in embryonic stem cells (13), response to injury in the retina and brain (14), and neoplastic transformation (15). However, the mechanisms responsible for the various phenotypes observed in vertebrates with IP deficiency or mutations remain elusive.

The proteolytic subunits of CPs are known to regulate gene expression, typically via cleavage of transcriptional regulators (16). Because CPs and IPs display distinct substrate preferences (4, 17), we hypothesized that they might have nonredundant effects on gene expression. This assumption was supported by preliminary gene expression microarray experiments showing that several transcripts are differentially expressed in wild-type (WT) and IP-deficient dendritic cells (DCs) (4). The goal of our work was therefore to evaluate the global impact of IPs on gene expression, discover its underlying mechanisms, and evaluate its in vivo relevance.

Based on multidimensional profiling of WT and Lmp7−/− Mecl1−/− (double knockout [dKO]) DCs, we report that IPs regulate the expression of >8000 transcripts in maturing DCs. The broad impact of IPs on gene expression is cell autonomous, mediated mainly at the transcriptional level, and likely involves major signaling pathways including IFN regulatory factors (IRFs), NF-kB, and STATs. Furthermore, even when engineered to present similar levels of a model epitope, dKO DCs were less effective than WT DCs in priming T cells in vivo. We conclude that IPs have pervasive effects on gene transcription and may thereby regulate fundamental cellular processes in DCs.

Materials and Methods

Mice and DC culture

Mice on a C57BL/6 or B6.SJL background were housed at the Institute for Research in Immunology and Cancer (IRIC) animal facility. Lmp7−/− Mecl1−/− (dKO) and Mecl1−/− mice were generously provided by Dr. T.A. Griffin from the Medicine College of the University of Cincinnati and Lmp7−/− mice by Dr. H.J. Feiling from the Institute of immunology.
were sorted for CD11c+CD86+CD45.1+ (WT) or CD11c+CD86+CD45.2+

anti-CD86 (allophycocyanin; BioLegend), anti-CD45.1 (allophycocyanin-

Biosciences) before staining with anti-CD11c (PECy7; BD Biosciences),

Harvested cells were treated for 20 min with Fc Block CD16/CD32 (BD

DCs, sorting was performed on DCs stimulated overnight with LPS.

coculture experiment of B6.SJL WT and C57BL/6 bone marrow–derived

maturation was induced with LPS (Sigma-Aldrich), as described (4). For

Bone marrow–derived DCs were generated from 8–12-wk-old mice, and

and natural contaminants. Reads that mapped two

targets were filtered using the TagMan technology with an ABI

Prism 7900HT system detection system (Applied Biosystems). Results

were analyzed with the SDS 2.2.2 software (Applied Biosystems). Primer

sequences are provided in Supplemental Table I. For transcript stability

assays, mature DCs were treated for 1, 3, 6, or 12 h with 5 μg/ml acti-
nomycin D (Sigma-Aldrich) or left untreated prior to harvest, extensive

washes, and RNA extraction. The Student t test was used to compare

RNA sequencing

RNA was extracted from four experimental replicates of WT and dKO

DCs stimulated for 0, 1, 2, or 6 h with LPS. Total RNA was isolated from

5 × 10^6 cells/sample with TRIzol RNA reagent (Life Technologies) as

recomended by the manufacturer. RNA-extracted samples were treated with

DNAse I and purified using the RNeasy Mini kit (Qiagen). Sample

quality was assessed using the 2100 Bioanalyzer RNA Nano chips (Agilent

Technologies). Transcriptome libraries were generated from 4 μg total

RNA using the TruSeq RNA Sample Prep Kit v2 (Illumina) following the

manufacturer’s protocols. Paired-end (2 × 100 bp) sequencing was per-

formed using an Illumina HiSeq2000 sequencer running TruSeq SBS v3

chemistry (Illumina) as previously described (18). Cluster density was

targeted at ~600,000–800,000 clusters/mm^2. A total of 16 transcriptomes

was sequenced per sequence. Data were mapped to the mouse refer-

cence genome (mm9) using the Casava 1.8.1 and Eland v2e mapping

database (http://www.ncbi.nlm.nih.gov/geo/) under accession number

GSE52616.

Differential gene expression analyses

Differential gene expression was determined using R v3.0.1 software (http://

www.r-project.org/) with the DESeq package (19). Comparisons were

made between WT and dKO conditions at each time point of LPS stim-

ulation or between untreated and LPS-stimulated cells for WT and dKO

independently. Significance was achieved when the p value adjusted for

multiple testing (Benjamini-Hochberg) was <0.1. An independent filtering

step, which removed ~40% of genes from the lowest quantile for overall

sum of counts for all conditions, was included to increase detection power

by the multiple testing algorithm. Genes removed through filtering were

found to achieve statistical significance; thus, this step reduced the

severity of the multiple testing adjustment without removing important

differentially expressed genes (DEGs).

The list of all 8104 genes that showed significant differential expression

between WT and dKO at any time point was analyzed with MeV (http://

www.tm4.org/mev.html) for clustering analyses of their kinetic of differ-

ential expression. Based on k-means clustering analyses, we determined

that 16 clusters were necessary and sufficient to adequately separate all

groups of genes with similar kinetics and that adding more clusters did not

improve significantly clustering quality.

Functional analyses of DEGs

DEGs from clusters 1–15 were uploaded into the Ingenuity Pathway

Analysis (IPA) software (Ingenuity Systems, http://www.ingenuity.com),

and upstream regulator analysis was performed from which we extracted

the most drastically affected molecules described as “transcriptional regu-

lators” (p < 0.05 with right-tailed Fisher exact test). The IPA’s z-score

algorithm was used to predict the direction of change for a given regulator

(increase [z-score ≥ 2] or decrease [z-score ≤ −2]) depending on the

dKO/WT ratio of expression of its target genes within a cluster, for each

time point (no LPS and 1-, 2-, and 6-h LPS). For each cluster, a map

was generated with any given regulator that had a p value ≤0.05 and a

z-score ≥2 or ≤−2 at one of the time points. Regulators passing these

two thresholds were highlighted. A gene ontology (GO) overrepresentation

analysis was performed with InnateDB (20), and lists of enriched bio-

logical processes were extracted (with adjusted p values for multiple

testing [Benjamini-Hochberg algorithm] <0.05).

Correlation between mRNA log_2 ratio and predicted activity

A correlation coefficient calculation was performed between the log_2 ratio

of mRNA differential expression and z-score of predicted activity of each

regulator for all time points of LPS stimulation. For regulators present in

more than one cluster, a correlation coefficient was calculated between the

log_2 ratio of expression and all combinations of z-score values found in

the different clusters. A strong correlation was defined as ≥0.5.

Chromatin immunoprecipitation

Immature DCs (no LPS stimulation) were crosslinked 10 min with 1% formaldehyde and then quenched 5 min with 1.25 M glycine. Crosslinked

samples were resuspended with cell lysis buffer (5 mM PIPES [pH 8], 85 m

M KCl, and 0.5% Nonidet P-40 [NP-40] with protease inhibitors). After 10

min on ice, samples were pelleted and resuspended with nuclei lysis buffer

(50 mM Tris [pH 8], 10 mM EDTA, and 1% SDS) for another 10 min.

Sonication was performed with a Bioruptor water sonicator (Diagenode) at

4°C (20 times 30 s on/off, setup high). Samples were centrifuged, supen-

natum transferred into a new tube, and 1/20 of samples kept as inputs. The

remaining of the samples was diluted two times with dilution buffer

(0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris [pH 8],

and 167 mM NaCl). Anti–histone 2B (H2B) (Abcam) or anti–ubiquitylated

H2B (ubH2B; Millipore) Abs were added for an overnight incubation at

5°C.

FIGURE 1. Gene expression changes are cell autonomous. (A) Quan-

titative RT-PCR experiments were performed on WT and dKO DCs cul-
tured either together or in separate Petri dishes. After overnight stimula-
tion with LPS, cells were harvested and sorted according to their CD45.1 (WT)

or CD45.2 (dKO) phenotype. (B) WT and dKO cells were harvested on
days 0 (bone marrow cells), 8 (immature DCs), and 9 (overnight LPS-
matured DCs) of culture and lysed for RNA extraction and RT-PCR

experiments. Left panel, Genes differentially expressed in both bone

marrow cells and DCs. Right panel, Genes differentially expressed in

immature and/or mature DCs only. Data are represented as mean ± SD of
three experimental replicates.
4˚C. PBS/BSA-washed magnetic Dynabeads Protein G (Life Technologies) were added for an additional 4 h. Samples were centrifuged, resuspended in dialysis buffer (2 mM EDTA, 50 mM Tris [pH 8], and 0.2% sarkosyl), and washed twice with dialysis buffer and four times with wash buffer (100 mM Tris [pH 9], 500 mM LiCl, 1% NP-40, and 1% sodium deoxycholate). Samples were eluted with freshly prepared elution buffer (1% SDS and 50 mM NaHCO3) for 10 min at 65˚C followed by 15 min of medium vortexing at room temperature. After centrifugation, elution steps were repeated once, and pooled samples as well as inputs were supplemented with 0.2 M NaCl and reverse-crosslinked overnight at 65˚C. Samples were precipitated with ice-cold ethanol, resuspended in TE (10 mM Tris-HCl [pH 8], 1 mM EDTA), and supplemented with 1 mg/ml RNAse A for 30 min at 37˚C and then with proteinase K buffer and 20 mg/ml proteinase K for 2 h at 45˚C.

**Microscopy**

DCs were stimulated or not overnight with 1 μg/ml LPS to induce maturation. As a negative control, one Petri dish was further supplemented with 10 μg/ml MG-132 (Calbiochem) for 2 h. Cells were then harvested, washed with fresh media, and plated onto 35-mm glass-bottom dishes (MatTek) with RPMI 1640 media without phenol red (Life Technologies), supplemented with 25 mM HEPES (Life Technologies). Cells were incubated for 2 h with 200 mM proteasome-bodipy fluorescent probe (generously provided by Dr. Huib Ovaa from the Netherlands Cancer Institute) or without compound for the unstained control. We found the proteasome-specific probe to be more reliable than Abs because it allowed us to have a reliable negative control: this consisted of a 2-h treatment with MG132, an unlabeled proteasome inhibitor, to block catalytically active sites prior to incubation with the proteasome-bodipy fluorescent probe. After washes, cells were stained for 45 min with Hoechst, further washed, and visualized by fluorescence microscopy. Imaging was performed on a Delta Vision Personal wide-field microscopy system (Applied Precision/GE Healthcare) consisting of an Olympus inverted IX71 fluorescence microscope equipped with a CoolSnap HQ2 camera (Photometrics) and a controlled environmental chamber. Cells were imaged at 37˚C with a 60 × PlanApo oil immersion objective (numerical aperture 1.42; Olympus) using DAPI and FITC filter sets (center/bandwidth in nm, DAPI excitation 360/40, emission 455/50; FITC excitation 490/20 emission 525/36) for Hoechst and Bodipy staining, respectively. Stacks of fluorescence images were acquired using Softworx software at 1 × 1 binning with 1 μm optical spacing (total 17 μm thickness), with a reference brightfield image taken at midsection. Image processing for visualization purposes, consisting of linear contrast adjustment, maximum intensity projection, and multichannel overlays, was carried out in ImageJ (National Institutes of Health).

**Immunoblot analyses**

DC whole-cell lysates were prepared and immunoblotted as previously described (4). Abs against histone 2A (H2A), H2B, and IRF7 were purchased from Abcam, calnexin and β-actin from Sigma-Aldrich, ubH2B from Millipore, and the rest from Cell Signaling Technology. To detect ubiquitylated proteins, cells were lysed in 20 mM TRIS-HCl, (pH 7.5), 10 mM EDTA, 100 mM NaCl, 1% NP-40, 0.1% SDS, 10 μM MG-132

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**FIGURE 2.** Relationship between proteasomes and differential gene expression. (A) RT-PCR experiments were performed on WT, Lmp7−/−, Mecl1−/−, and dKO DCs stimulated overnight with LPS. Data are represented as mean ± SD of three to seven experimental replicates. (B and C) RT-PCR experiments were performed on WT and dKO DCs pretreated for 2 h with graded concentrations of epoxomicin and then stimulated for 6 h with LPS. Data are represented as mean ± SD of three experimental replicates. See also Supplemental Fig. 1. Epx, epoxomicin.
(Calbiochem), 10 mM NEM (Sigma-Aldrich), and a protease inhibitor mixture (Complete; Roche), as described (21). Immunoblot was performed with anti-ubiquitin from Santa Cruz Biotechnology and anti–poly-Ub (FK1 clone) from Enzo Life Sciences. The Student t test was used to compare means.

Native gel immunoblotting

Sample preparation for native gel immunoblotting was performed as described (22). Then, 30 μg dialyzed DC lysate was supplemented with 0.05% of NativePAGE G-250 sample additive, then run on a 3–12% NativePAGE Bis-Tris gel (Life Technologies) in NativePAGE running buffer (Life Technologies) at 4°C for 15 min at 100 V, and then increased to 180 V for 4 h. Cathode buffer was supplemented with 0.02% (for the first 15 min of run) or 0.002% (for the last 4 h of run) of Coomassie G-250. For immunoblot with anti-LMP2, gels were briefly boiled in SDS-PAGE Laemmli 1× buffer. Proteins were then transferred to a polyvinylidene difluoride, blocked in 5% milk/TBS, and immunoblot analysis was performed with anti-PSMA3 (Cell Signaling Technology), anti-PSMD4 (Cell Signaling Technology), or anti-LMP2 (Abcam).

Adoptive transfer, immunization, and evaluation of OT-1 T cell response

CD8+ T cells from female OT-1 (CD45.2+) mice were CFSE labeled (Molecular Probes; see manufacturer’s instructions) as previously described (23), and 10^6 labeled cells was injected i.v. into female B6.SJL (CD45.1+) mice. Twenty-four hours later, recipients were immunized i.v. with 1×10^6 male WT or dKO DCs stimulated overnight with LPS and pulsed with OVA257–264 peptide (2 μM) (24). Levels of K b/SIINFEKL at the surface of DCs were monitored by flow cytometry using a PE-conjugated anti-Kb/SIINFEKL Ab (eBioscience). Three and 5 d (or 45 d for the no recall control) following immunization, spleens were harvested, and T cell response was analyzed by flow cytometry. Alternatively, a second immunization with WT or dKO DCs was performed at day 30, and spleens were harvested on day 32 (day 2 postrecall). For Annexin V staining (measurement of apoptosis), splenocytes were incubated with 5 μl Alexa Fluor 350 Annexin V (Life technologies) in 100 μl Annexin binding buffer for 15 min at room temperature. After 10 min, 1.25 μg/ml 7-aminoactinomycin D was added. OT-1 T cells were identified using anti-CD8a (PE-Cy7; BD Biosciences) and anti-CD45.2 (PE; BD Biosciences). Flow cytometry analyses were done on a BD LSR II flow cytometer using FACSDiva (BD Biosciences) and FCS v.3.0 (De Novo Software) softwares. To calculate the frequency of responding OT-1 T cells, division peaks (as determined by CFSE intensity) were labeled from 0 to n. Making use of the experimentally determined number of events under each CFSE fluorescence peak, the absolute and the relative frequency of OT-1 precursors that responded to SIINFEKL-pulsed DCs was calculated as previously described (25, 26): the sum of the extrapolated responding precursors for division cycles from 1 to n allowed us to determine the relative or the absolute size of the precursor sample pool that responded.

Results

The effect of IPs on gene expression is cell autonomous

We generated DCs by culturing bone marrow in the presence of GM-CSF (4), which gives rise to a largely homogenous population of CD11c<sup>+</sup>CD11b<sup>+</sup>B<sub>2</sub>2<sup>+</sup> conventional DCs (cDCs) (27). In previous microarray experiments, we identified 226 transcripts that were differentially expressed in WT versus dKO DCs (4). This differential expression was cell autonomous because it persisted whether DCs

![Figure 3](http://www.jimmunol.org/)

**FIGURE 3.** IPs do not globally affect mRNA stability or maturation. Quantitative RT-PCR experiments were performed following actinomycin D treatment. Mean mRNA t<sub>1/2</sub> (indicated in each graph) was calculated in WT (blue) and dKO (red) DCs for transcripts previously showed to be overexpressed (A) or downregulated (B) in WT compared with dKO DCs. Data are represented as mean ± SD of three to seven experimental replicates. (C) RT-PCR experiments were performed on DNase-treated RNA samples from WT and dKO DCs with primers designed to target exon–exon junctions (mature mRNAs) or primers either spanning exon–intron junctions or within introns (unprocessed mRNAs). Data are represented as mean ± SD of three to seven experimental replicates.
derived from WT and dKO bone marrow cells were cultured separately or together (Fig. 1A). Furthermore, RT-PCR analyses at different time points of DC culture revealed two major differential expression patterns between WT and dKO cells. A first group of genes was already differentially expressed in bone marrow precursors and remained as such throughout DC differentiation and maturation (Fig. 1B, left panel; all genes differed significantly between WT and dKO at all time points). The second group consisted of genes that were affected only in mature DCs (Bmp2k, p < 0.05 at t = 9 d) or in both immature and mature DCs (C1qb and Snf1lk with p < 0.05 at days 8 to 9) (Fig. 1B, right panel). These results show that transcriptomic variations linked to IPs can be differentiation stage dependent.

Relationship between proteasome subunits and differential gene expression

IP subunits are cooperatively incorporated into nascent proteasomes, and the inclusion of catalytic subunit LMP2 is facilitated by the incorporation of LMP7 and MECL1 (28). Accordingly, we detected only low levels of LMP2/β2/β5 mixed proteasomes in dKO DCs (Supplemental Fig. 1A), which thus contained mainly CPs. RT-PCR experiments on DCs isolated from WT, Lmp7−/−, Mecl1−/−, and dKO mice (Fig. 2A) further showed that for the majority of genes tested (11 out of 13), the absence of both LMP7 and MECL1 was required to affect gene expression. A 2-h pretreatment with the selective and potent inhibitor of both CPs and IPs epoxomicin before LPS stimulation eliminated differential expression of Bmp2k, Cxcl11, and Apobec3 in WT versus dKO DCs (Fig. 2B), but not of Dlc1 and Gdpd3 (Fig. 2C). We therefore conclude that differential expression of some but not all genes is tightly linked to proteasomal degradation. Of note, proteasome inhibition can negatively affect transcription via endoplasmic reticulum stress induction. However, this was not the case in our experimental conditions: mRNA level of Bip, a marker of endoplasmic reticulum stress (29), was not increased in epoxomicin-treated compared with control DCs (Supplemental Fig. 1C).

Quantitation of proteasome levels using non-denaturing gel analysis of PSMA3, a 20S core subunit present in both IP and CPs, and PSMD4, a 19S regulatory subunit, showed that WT and dKO DCs contained similar amounts of 20S and 26S proteasomes (Supplemental Fig. 1B). Furthermore, probing active proteasomes by fluorescence microscopy, using a catalytic inhibitor coupled to a fluorochrome, revealed that CPs and IPs presented similar distribution profiles (Supplemental Fig. 1D). Therefore, although differences in gene expression between WT and dKO DCs are at least partly linked to proteasomal activity, these cannot be ascribed to differences in the total abundance or localization of proteasomes and are likely due to differences in proteasome composition.

**FIGURE 4.** IPs have a broad impact on the transcriptional program of maturing DCs. (A) Venn diagram showing the 8104 DEGs in RNA-sequencing experiments. (B) Differential expression of genes between WT and dKO DCs untreated or stimulated with LPS for 1, 2, or 6 h. Red dots represent DEGs with a significant adjusted p value (p < 0.1, Benjamini-Hochberg algorithm). (C) All 8104 DEGs were clustered into 15 different groups as a function of their expression pattern using k-means clustering. Each heat map represents one cluster with color-coded fold changes at each time point of LPS stimulation, with the number of genes included written underneath. Based on RNA-sequencing data submitted to the Gene Expression Omnibus database under accession number GSE52616.
IPs do not affect mRNA stability and have a minimal influence on mRNA maturation

Because factors involved in both mRNA stability and splicing are regulated by ubiquitylation and proteasomal degradation (30–32), we wished to compare the stability of transcripts overexpressed in WT or in dKO DCs following treatment with actinomycin D (Fig. 3A, 3B). We found that transcript \( t_{1/2} \) were similar between WT and dKO DCs, showing that IPs have no impact on mRNA stability. To examine the effect on mRNA maturation, we compared by RT-PCR the level of both spliced (mature) and unspliced (unprocessed) transcripts derived from differentially expressed genes (Fig. 3C), using primer pairs spanning exon–exon junctions or primer pairs within introns or spanning exon–intron junctions (33). For \( Rpt29 \) and \( Hebp1 \), IP deficiency, respectively, decreased or increased mature mRNA abundance but had no impact on the unprocessed transcripts (Fig. 3C, right panel). However, for 14 out of 16 transcripts (87%), absence of IP subunits resulted in parallel upregulation or downregulation of both mature and unprocessed mRNAs (Fig. 3C, left panel). Overall, these results strongly suggest that differences in transcripts abundance between WT and dKO DCs: 1) do not involve variations in mRNA stability; 2) result from differential interactions with the pre-mRNA splicing and maturation machinery in a few cases (~13%); and 3) must therefore be regulated at the level of mRNA synthesis in most cases (~87%).

IPs have a broad impact on the transcriptional program of maturing DCs

To evaluate the overall impact of IPs on the transcriptional program and the biology of DCs, RNA-sequencing analyses were performed at different time points of LPS-induced DC maturation (0, 1, 2, and 6 h). By comparing the transcriptome of WT and dKO DCs at each time point, we found a total of 8104 differentially expressed genes (DEGs, Fig. 4A). Out of these, 19% (1570) were affected at all stages of maturation, 38% (3107) were altered at only one specific time point, and 43% were affected at two to three time points (Fig. 4A). IPs had a greater impact on mature than on immature DCs (Fig. 4A, 4B), and, strikingly, 64.7% of all the genes affected by LPS in WT cells were differentially expressed in WT versus dKO DCs (7,419 out of the 11,455 DEGs affected after 1, 2, or 6 h of LPS, data not shown). We conclude that: 1) the impact of IPs on the transcriptome is highly dependent on the maturation state of DCs; and 2) a great proportion of genes modulated by LPS are regulated by IPs. Because we did not perform coculture experiments between WT and dKO DCs (as in Fig. 1), we could not formally assess whether some variations in the transcriptional landscape were due to autocrine or paracrine secretion of cytokines.

Given the complexity of gene expression variations, we stratified our analyses by focusing on groups of genes that had similar kinetics of differential expression over time. The total 8104 DEGs were unbiasedly separated into different groups, using the k-means clustering algorithm, as a function of their dKO/WT fold change at each time point (Fig. 4C). We found 15 different kinetic patterns, each including from 205 to 1278 genes. Different groups of DEGs were affected at early (1-h LPS, clusters 4 and 12), intermediate (2-h LPS, clusters 3 and 11), or late time points (6-h LPS, clusters 1 and 10), and others were modulated at more than one time point. The complexity of the transcriptional cascade regulated by IPs and the emergence of discrete gene clusters at various time points suggest that IPs may control transcription through distinct mechanisms at different stages of DC maturation. We surmised that such broad transcriptomic variations could result from two non–mutually exclusive mechanisms: variations in histone ubiquitylation and proteolysis of transcription factors.

Variations in histone monoubiquitylation cannot explain the impact of IPs on the transcriptome

The amount of free monoubiquitin in cells is very small. Therefore, upon activation of the ubiquitin–proteasome system, the ubiquitin supply is largely obtained from ubiquitylated histones (34). Hence, we speculated that widespread changes in the pool of ubiquitylated histones, which affects transcriptional activity (16), might explain the broad impact of IPs on the transcriptome. Although no differences were observed in total levels of ubH2A or total ubiquitylated proteins at any stage of DC maturation, dKO immature DCs contained more ubH2B than WT DCs (Fig. 5A, Supplemental Fig. 2A). This discrepancy disappeared after LPS treatment. Nonetheless, in immature cells, following chromatin immunoprecipitation of ubH2B and quantitative PCR amplification of coding regions of a subset of DEGs, we observed no differences in ubH2B enrichment between WT and dKO DCs (Fig. 5B). In line with this, although unstimulated *Lmp7*+/− DCs contained increased amounts of ubH2B (Supplemental Fig. 2B), they did not show changes in gene expression relative to WT DCs (Fig. 2A). We conclude that increased levels of ubH2B were not responsible for discrepancies in the transcriptomic profile of WT versus dKO DCs.

**FIGURE 5.** The broad gene expression changes cannot be explained by variations in histone monoubiquitylation. (A) Nuclear extracts were prepared from WT and dKO DCs stimulated for the indicated time duration with LPS and immunoblotted with Abs against total or monoubiquitylated H2A or H2B. Histograms represent the mean ± SD of five experimental replicates. (B) Chromatin immunoprecipitations (ChIP) were performed using an anti-ubH2B Ab in WT and dKO immature (LPS-unstimulated) DCs. Precipitated DNA was subjected to quantitative PCR analysis with primers targeting genes found differentially expressed at indicated time points. A parallel reaction was performed with anti-H2B for normalization. Data are represented as mean ± SD of two experimental replicates. See also Supplemental Fig. 2A and 2B. Ctrls, controls.
partitioned into k-means clusters (Fig. 4C). For each cluster, a map was generated including every transcriptional regulator that had a z-score $\geq 2$ or $\leq -2$ (activity predicted to be respectively increased or decreased in dKO), and significant enrichment ($p \leq 0.05$), for at least one of the time points (Fig. 6A). A total of 155 regulators were identified, of which 88 were nonredundant: 53 were predicted to be differentially active (i.e., to target DEGs) in a single cluster, and 35 were predicted to be differentially active in $\geq 2$ clusters. These included STAT1, IRF7, IRF1, and STAT4. Protein–protein interaction analysis using STRING (35) also showed that members of the IRF and STAT families formed nodes of strong connectivity within regulator-rich clusters 1, 6, and 7 (data not shown), placing them as likely master regulators differentially affected by CPs and IPs.

To validate IPA-based predictions concerning the activity of IRF, STAT, and NF-κB pathways, we analyzed the expression of some of their members by immunoblotting. RELA (p65), the transcriptional activator of the canonical NF-κB pathway, showed increased predicted activity in mature dKO DCs (6 h) for clusters 1 and 7. In line with this, protein levels of IκBα, the pathway inhibitor, were decreased at 2–6 h in dKO DCs, coinciding with an increased ratio of p-IκBα/total IκBα at 0 h ($p = 0.01$) (Fig. 6C, Supplemental Fig. 2C). In accordance with predictions, IRF3 and IRF7 levels were also markedly reduced in immature dKO DCs, as well as STAT1, STAT5, and STAT6 at all time points and STAT3 for up to 1 h of LPS treatment (Fig. 6B, 6C, Supplemental Fig. 2C). For STAT3, STAT5, and IRF3, ratios of activated (phosphorylated) over total protein abundance were similar in WT and dKO DCs, suggesting that IPs regulated protein abundance but not phosphorylation efficiency. Together, IPA analyses and immunoblotting experiments strongly suggest that, at least in DCs, IPs have a nonredundant effect on key signaling pathways including STATs, IRFs, and NF-κB.

**IP deficiency affects key DC genes and functions**

As a complement to our analyses of signaling pathways, we sought to identify specific genes and biological processes that were most affected by IPs. We found that none of the top DEGs between

![FIGURE 6. STAT, IRF, and NF-κB pathways are perturbed in IP-deficient DCs.](http://www.jimmunol.org/)

(A) For each k-means cluster (Fig. 4C), IPA software was used to identify regulators of transcription, along with their number of target genes, significantly enriched ($p < 0.05$ with Fisher algorithm) and predicted to be more activated in dKO (z-score $\geq 2$) or in WT (z-score $\leq -2$) (both designated in red) for at least one time point of LPS stimulation. No regulator was found significantly enriched in cluster 12. Immunoblot analyses of whole-cell lysates from DCs either deprived of FBS for 3 h (B) or left unstarved (C) and then stimulated with LPS for the indicated time. See also Supplemental Fig. 2C.)
WT and dKO DCs (≥10-fold difference) were involved in DC function (Table I) (36–44). Genes expressed at low levels (≤0.1 reads/kb/million mapped reads) were excluded from this analysis. Interestingly, the six transcripts overexpressed in WT DCs encode proteins that have a positive effect on DC function (e.g., CD207 and TLR11). By contrast, the top dKO-overexpressed genes encode proteins associated with negative regulation of the immune response (e.g., MASP2 and GPD3).

To obtain a broader perspective of the processes affected by IP deficiency at different time points, we performed GO-term enrichment analyses for each of the 15 gene clusters displayed in Fig. 4C (Fig. 7A). Two main points can be made from these analyses. First, GO terms enriched in WT versus dKO DCs encompassed housekeeping processes that are tightly regulated during DC activation (cell cycle, transcription and translation, protein transport, and ubiquitylation) (45) and more specific immune roles (immune and inflammatory response). Second, the maturation programs of dKO and WT DCs did not progress simultaneously. Thus, genes related to cell-cycle regulation were enriched early in WT DCs and late in dKO DCs. Furthermore, the kinetics of genes associated to immune-related biological processes were particularly complex, and groups of transcripts followed at least four discrete patterns upon LPS stimulation (data not shown): 1) overexpression at all time points in dKO compared with WT DCs (e.g., Ccl4, Il6, and Cxcl11); 2) more prolonged upregulation in dKO than WT DCs (e.g., Cxcl10); 3) delayed response in dKO DCs (e.g., Ccl5); and 4) overexpressed in WT DCs at all time points (e.g., Ccl22, Ccr7, and Tnfsf4). Collectively, top differentially expressed genes and GO-term enrichment analyses strongly support the idea that IPs have profound and pleiotropic effects on DC maturation and function. Furthermore, relative to WT DCs, IP-deficient DCs showed defective or delayed expression of genes involved in DC maturation and function (Fig. 7A, Table I). Consistent with this, we observed that LPS-induced CD86 upregulation at the cell surface, a hallmark of DC maturation, was delayed in IP-deficient DCs (Fig. 7B).

**IP-deficient DCs are phenotypically more similar to macrophages than WT DCs**

Exhaustive analyses of the transcriptional landscape of cDCs and macrophages have yielded fundamental insights into the biology of these two subsets of mononuclear phagocytes. By analyzing the transcriptome of our DC populations with regards to recently uncovered key features of these cell populations, we observed that five genes involved in cDC development—Baf53, Irf4, Irf8, Relb, and Bcl6 (46, 47)—were overexpressed slightly, but significantly, in WT relative to IP-deficient DCs (Fig. 7C). On the contrary, Mafb, a key element of the core transcriptional signature of macrophages (46), was overexpressed in IP-deficient DCs. To further explore the relationship between our DC populations and macrophages, we used the robust and comprehensive data generated by the Immunological Genome Project, which has defined core signatures for these cell types (48, 49). Out of the 24 transcripts composing the core cDC signature, 2 were overexpressed in dKO DCs, and 18 were overexpressed in WT DCs (Table II). Among the 39 transcripts defining the macrophage signature, 26 were overexpressed in dKO DCs, and 1 was overexpressed in WT DCs (Table II). Overall, comparing cDC and macrophage signature genes overexpressed in WT versus dKO DCs provided compelling evidence that IP-deficient DCs are more similar to macrophages than WT DCs (p < 0.001, Fisher exact test).

**dKO DCs are functionally deficit in vivo**

If the transcriptomic differences between WT and dKO DCs are biologically relevant, they should affect the quintessential role of DCs: Ag presentation to T lymphocytes (50, 51). We therefore compared the T cell–priming ability of WT and dKO DCs presenting similar cell-surface density of the model epitope SIINFEKL (Fig. 7E), taking into account the fact that dKO DCs are not immunogenic for WT mice (4). We found that adoptively transferred OT-1 T cells, which recognize H2Kb/SIINFEKL, expanded more extensively 3–5 d following priming with WT than with dKO SIINFEKL-pulsed DCs (Fig. 7D). This difference could be explained by two factors: an increased frequency of responding T cells and decreased proportion of apoptotic T cells following immunization with WT than dKO DCs (Fig. 7F, 7G). Interestingly, the decreased capacity of dKO DCs to prime T cells was maintained when mice were challenged with a second DC immunization at day 30 (Fig. 7D). Hence, WT DCs were more effective than dKO DCs for priming both naive and memory CD8 T cells, even when both DC populations were engineered to present similar epitope density.

### Discussion

The dominant paradigm holds that the ultimate nonredundant role of IPs versus CPs is to generate a highly diverse MIP repertoire. The present work shows that nonredundant roles of IPs go well beyond MIP generation. We report that IPs regulate the expression

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**Table I. Selected top DEGs between WT and dKO DCs**

<table>
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<tr>
<th>Genes</th>
<th>Log2 dKO/WT (t = 6 h LPS)</th>
<th>Protein Function</th>
<th>Reference No.</th>
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<tr>
<td>Cd300e</td>
<td>−4.48</td>
<td>Induces cytokine production (IL-12 and TNF-α) from stimulated DCs</td>
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<td>Tlr11</td>
<td>−3.30</td>
<td>Induces cytokine production (IL-12 and TNF-α) from stimulated DCs and binds to</td>
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<tr>
<td></td>
<td></td>
<td>parasites</td>
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<tr>
<td></td>
<td></td>
<td>Binder of carbohydrates at the surface of viral envelopes</td>
<td>(38)</td>
</tr>
<tr>
<td>Ccl207 (Langerin)</td>
<td>−6.44</td>
<td>Involved in endocytosis and Ag uptake</td>
<td>(39)</td>
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<tr>
<td>Siglech</td>
<td>−4.14</td>
<td>Promotes DC migration from the epidermis to lymph nodes and regulates cell–cell interactions</td>
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<td>Gpr77</td>
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<tr>
<td>Masp2</td>
<td>3.66</td>
<td>Attenuates DC maturation and response to proinflammatory cytokine production</td>
<td>(42)</td>
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<tr>
<td>Gdpd3</td>
<td>5.31</td>
<td>Putative negative regulator of DC migration</td>
<td>(43)</td>
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<td>Cxcl11</td>
<td>3.56</td>
<td>When bound to CXCR7, inhibits migration of DCs in response to CXCL4</td>
<td>(44)</td>
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of >8000 transcripts in DCs. Of note, IPs may affect the expression of additional genes redundantly with CPs, but our study design was poised to selectively identify genes differentially regulated by IPs and CPs.

The impact of IPs was cell autonomous and, at least for genes tested in Fig. 2, required deletion of both Mecl1 and Lmp7 subunits. Consistent with the fact that incorporation of LMP2 in proteasomes is impaired in the absence of MECL1 and LMP7, we found in our dKO DCs only low amounts of LMP2-containing mixed proteasomes (in this study, LMP2/b2/b5). Hence, dKO mice used in this study were practically deficient for the three IP catalytic subunits. That we observed gene expression changes in dKO DCs but not in DCs deficient only for MECL1 or LMP7 could be explained by two alternatives: 1) MECL1 and LMP7 have redundant roles; or 2) they have different but synergistic effects on gene expression. Nevertheless, it is formally possible that some transcripts that were not included in Fig. 2 might be differentially expressed in WT DCs and DCs lacking a single IP.

**FIGURE 7.** Presence of IPs is important to maintain DC functional integrity. (A) InnateDB was used to identify biological processes significantly enriched in genes associated to each k-means cluster (Fig. 4C). Boxes represent clusters with a particular kinetic of differential expression between WT and dKO (cluster identification numbers are shown in each box). The size of a box is representative of the time frame for differential expression. Biological processes indicated had the highest p value, or were the most representative, among significantly enriched GO terms for each cluster. Clusters 4 and 15 were not represented either because no biological process was found significantly enriched or because no pattern was found within enriched terms. (B) Percentage of CD86+ cells among total CD11c+ DCs during LPS stimulation; mean ± SD of four experimental replicates. (C) Relative expression of core genes involved in cDC development or part of the transcriptional signature of macrophages (Mø). Blue and yellow colors indicate overexpression in WT and dKO DCs, respectively. (D) CFSE-labeled OT-1 T cells were injected into WT mice prior to immunization with LPS-treated and SIINFEKL-pulsed WT or dKO DCs. Spleens were harvested 3 or 5 d postimmunization or 2 d after a second immunization on day 30. Absolute numbers of OT-1 T cells and percentages of OT-1 among total splenocytes are presented as the mean ± SD of 4–13 experimental replicates. (E) Kb/SIINFEKL levels measured by flow cytometry at the surface of WT and dKO DCs pulsed or not for 3 h with the model SIINFEKL (OVA257–264) peptide. Data are represented as mean ± SEM of six experimental replicates. (F) The absolute and relative size of OT-1 T cell populations that responded to SIINFEKL-pulsed DCs were calculated from CFSE labeling profiles on day 3 postimmunization. Data are represented as mean ± SD of six experimental replicates. (G) Proportion of dead and apoptotic OT-1 T cells on day 3 after priming with WT or dKO DCs. Data are represented as mean ± SD of seven experimental replicates.
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The gene signature of cDCs and macrophages was retrieved from Miller et al. (48) and Gautier et al. (49). The expression level of these genes was compared between WT and dKO DCs based on RNA-sequencing data (available in the Gene Expression Omnibus under accession number GSE52616). Values are included only for one time point: no LPS for the cDC signature and 6-h LPS for the macrophage signature. Similar results were observed at other time points (data not shown).

*p < 0.0001.

NA, not applicable.
subunit. Such differential expression could be due to a loss of function (of IPs) or a gain of function linked to the presence of increased amounts of mixed proteasomes in DCs deficient for a single IP subunit.

In various cell types, including DCs, ~80% of the observed variance in mRNA levels is shaped by transcription rates rather than posttranscriptional regulation (52). Accordingly, our data strongly suggest that IPs shape the transcriptome mainly via regulation of transcription factors and not by affecting mRNA stability. Nonetheless, expression of a few transcripts (Rpl29 and Hepb1) reflected an impact of IPs on pre-mRNA processing events (Fig. 3). It is well recognized that CPs control the activity of most transcription factors (16). However, the breadth of nonredundant effects of IPs on the transcriptome was somewhat unanticipated.

Treatment of WT and dKO DCs with a selective proteasome inhibitor swiftly abolished differences in the abundance of Bmp2k, Cxcl11, and Apobec3 transcripts (Fig. 2B). These cases probably involved proteolysis of short-lived transcription factors or their regulators. Contrarily, differential abundance of Dicl1 and G6pd1 was not tightly linked to proteasomal activity (Fig. 2C), probably because it was regulated by proteolysis of transcription factors with longer $t_{1/2}$, which would be missed within the time frame of our proteasome-inhibition experiment.

Clustering of the genes according to their kinetics of differential expression showed that the imprint of IPs on gene expression was extensive, dynamic, and affected all stages of DC maturation. Nonetheless, the impact was greater in mature than in unstimulated DCs. Thus, as much as 64.5% of genes induced or repressed by LPS stimulation were regulated by IPs. With $>8000$ DEGs, the effects of IPs were obviously complex, but IPA analysis suggests that a major proportion of DEGs was regulated by 88 unique transcription regulators, many of which were related to the STAT, IRF, and NF-$\kappa$B pathways. Immunoablotting confirmed the variable abundance of regulators’ protein levels in WT and dKO. Because IPs and CPs show distinct processing kinetics (53) and cleavage preferences (4, 54), we surmise that STATs, IRFs, and cleavage preferences (4, 54), we surmise that STATs, IRFs, and NF-$\kappa$B are processed at different rates in WT DCs (containing both CPs and IPs) versus dKO DCs (containing almost exclusively CPs), leading to alterations in the transcriptome profile. Notably, altered IxBa degradation has previously been linked to mixed proteasomes in Lmp2-/- B lymphocytes (11). Additionally, WT DCs overexpressed genes associated to core cDC signature, whereas dKO DCs overexpressed various macrophage-specific genes, suggesting that dKO DCs are more similar to macrophages than WT DCs.

Previous studies have shown that IP-deficient DCs were inefficient in priming of CDS T cell responses (5). Heretofore, the role of IPs in MIP processing has been held primarily responsible for this impaired ability. We show in this study that, even when engineered to present the same epitope density as WT DCs, dKO DCs remained defective APCs (Fig. 7D–G). Relative to WT DCs, dKO DCs showed two defects: they primed limited number of naive and memory T cells, and these primed T cells were unduly susceptible to apoptosis. Further work will be necessary to uncover the mechanistic underpinnings of these defects. Nonetheless, they clearly show that the functional role of IPs in DCs goes well beyond MIP production. They also suggest that the level of IP expression, which is maturation stage dependent in DCs (55), may be relevant to DC-based vaccination trials (56).

The imprint of IPs on the transcriptome of DCs was not limited to immune genes. Indeed, in the present work, IPs were found to regulate genes involved in numerous fundamental processes including transcription, translation, protein economy, and cell-cycle regulation. In various models, IPs have been associated to maintenance of stemness, cell differentiation, response to injury, and neoplastic transformation (12–15). We therefore speculate that, if found in other cell types, the dramatic effect of IPs on the transcriptional landscape could explain the various immune and non-immune phenotypes observed in vertebrates with IP deficiency or mutations.

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Disclosures
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